METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

5   The applications claims the benefit of U.S. Provisional Application No. 60/167,423, filed November 24, 1999.

FIELD OF THE INVENTION

The invention relates to a method for freezing sperm selected for a particular characteristic, as well as to a frozen selected sperm sample and methods of using such a sample.
10   The invention is particularly useful for preserving sex-selected sperm.

BACKGROUND OF THE INVENTION

Over half a century ago, artificial insemination was introduced in the United States as a commercial breeding tool for a variety of mammalian species. Although artificial insemination was initially limited to regions relatively close to the site of sperm collection, advances in the
15   cryopreservation and storage of sperm have facilitated widespread distribution and commercialization of sperm intended for artificial insemination or in vitro fertilization.

Further improvements in mammalian sperm collection, selection, cryopreservation, storage, and handling techniques have enhanced the ability of breeders to produce animals having desired traits. For example, advances in selection of mammalian sperm based on slight
20   differences in physical characteristics has made it possible to separate sperm based on sex-type, that is, to select for cells containing either the X or Y chromosome. This technique allows the breeder to manipulate the relative percentage of X- or Y-type sperm in a sample and thereby determine offspring sex. The ability to select sperm based on sex-type or any other desirable characteristic provides an important tool for accelerating genetic progress, increasing production
25   efficiency, and achieving greater flexibility in livestock management. Full exploitation of this tool, however, depends on the ability to freeze and store selected sperm.

A variety of methods are available for selecting cells; however, the selection and subsequent processing of sperm presents unique challenges because sperm are incapable of DNA repair and because of sperm morphology. Each sperm has an acrosome overlying the head and a
30   tail, which are important for fertility and which are relatively susceptible to physical injury. In

1

addition, sperm fertility decreases with increasing time between collection and use. As most of the available selection methods involve physical stresses and take time, selected sperm are typically somewhat compromised compared to non-selected cells. Fertility may be further reduced if the selection technique involves significant dilution. It has been suggested that this

5   "dilution effect" may be due to the loss of protective components in seminal plasma.

Flow cytometry is a particularly efficient selection method that has been employed for sorting sperm by sex-type. However, sorted sperm are subject to stresses beyond those normally encountered in standard artificial insemination or in vitro fertilization protocols. In particular, flow cytometry is time consuming, and, because of the physical constraints of flow cytometers,

10   sperm must be diluted for sorting to levels that are not optimal for storage (usually to on the order of 10^5-10^6/ml). Furthermore, sorted sperm intended for artificial insemination must be concentrated so that conventional packaging and delivery equipment can be used. The need for a concentration step thus exposes already somewhat compromised sperm to additional physical stresses.

15   The freezing of sperm also invariably reduces fertility, motility, and/or viability, and, although techniques for freezing unselected sperm are well known, no technique for cryopreservation of selected sperm has been described.

SUMMARY OF THE INVENTION

The present invention provides a method of cryopreserving sperm that have been selected

20   for a specific characteristic. The method is particularly useful for cryopreserving sperm selected by a method that results in dilution of the sperm, since the method provides for the isolation of sperm from a selected sperm sample, followed by addition of a final extender to the isolated sperm to produce a suspension having a desired concentration of sperm. In a preferred embodiment, the method is employed to freeze sex-selected sperm. Although the

25   cryopreservation method of the invention can be used to freeze sperm selected by any number of selection methods, selection using flow cytometry is preferred.

The present invention also provides a frozen sperm sample that has been selected for a particular characteristic, such as sex-type. In preferred embodiments, the frozen sperm sample

30   includes mammalian sperm, such as, for example, human, bovine, equine, porcine, ovine, elk, or

2

bison sperm. Also within the scope of the invention is a container including a frozen sperm sample according to the invention.

The frozen selected sperm sample can be used in a variety of applications. In particular, the sample can be thawed and used for fertilization. Accordingly, the invention also includes a method of using the frozen selected sperm sample for artificial insemination or in vitro fertilization.

DETAILED DESCRIPTION OF THE INVENTION

The present invention allows cryopreservation of sperm that have been selected for a particular characteristic, facilitating storage and/or shipment of selected sperm samples to sites distant from the collection site. Thawing yields viable sperm that can be used in procedures such as artificial insemination ("AI") and in vitro fertilization ("IVF"). This result was surprising because of the well-documented fragility of sperm. Prior researchers had demonstrated that the stresses associated with various selection methods or with cryopreservation resulted in significant losses in fertility and/or viability. The present inventors have demonstrated, for the first time, that pregnancies can be achieved with sperm that have been selected and then frozen.

The invention represents an important advance in livestock management, where selection of sperm for use in such procedures can be used to increase the production of offspring having desirable traits. For example, selection to obtain sperm carrying either the X or the Y chromosome allows control over offspring sex, which is advantageous for producers of animals such as dairy or beef cattle. Sex selection also finds application in breeding valuable (e.g., show or race horses) or endangered animals. The ability to freeze selected sperm, which the invention provides, will enable widespread use of such selection methods to, e.g., increase livestock production efficiency as well as quality.

Definitions

The term "acrosome" or "acrosomal cap" refers to the cap that covers the anterior half of the head of sperm and that contains enzymes necessary for ovum penetration.

The term "sex-type" refers to the type of sex chromosome present in the sperm (i.e., the X or Y chromosome).

3

The term "capacitation" refers to the specific changes a sperm undergoes to develop the capacity to fertilize ova, such as enzymic changes on the surface of the acrosome that lead to release of acrosomal enzymes that facilitate penetration of the sperm into the ovum.

As used with reference to sperm, the term "cryoprotectant" refers to a molecule that

5   protects sperm during a freeze-thaw cycle, promoting survival and retention of fertilizing capacity.

The term "dilution effect" refers to the rapid decline in motility and/or viability of sperm when highly diluted.

As used herein, the term "selection" refers to a method whereby a sample is subdivided

10   based on presence or absence of a specific characteristic (unless context dictates otherwise). Thus, a "selected sperm sample" is a sample obtained by subjecting a source sample to selection for the specific characteristic. A selected sperm sample is therefore enriched, relative to the source sample, in sperm having the specific characteristic.

The term "sorting" is used herein to describe a selection method carried out using a

15   fluorescence-activated cell sorter (FACS).

The term "extender" refers to any medium that tends to preserve sperm viability. The term "extension" refers to the dilution of sperm with extender.

The term "initial extender" refers to a medium used to extend sperm prior to the isolation step of the method of this invention.

The term "final extender" refers to a medium used to extend sperm prior to the freezing

20   step of the method of this invention.

An "organic substance" in an extender described herein is any organic substance that tends to reduce cold shock and preserve fertility of sperm.

An "energy source" in an extender described herein is any substance or substrate that

25   sperm can utilize for cell maintenance and/or motility.

The term "osmolality," as used herein, is a measure of the osmotic pressure of dissolved solute particles in a an aqueous solution (e.g., an extender). The solute particles include both ions and non-ionized molecules. Osmolality is expressed as the concentration of osmotically active particles (i.e., osmoles) dissolved in 1 kg of water.

30

4

Cryopreservation Method

The invention provides a method of cryopreserving selected sperm includes the following steps:

(1)   obtaining a selected sperm sample;

(2)   cooling the selected sperm sample;

(3)   isolating sperm from the selected sperm sample;

(4)   adding final extender to the isolated sperm to produce a suspension of sperm; and

(5)   freezing the suspension of sperm.

Obtaining a Selected Sperm Sample

The first step in the cryopreservation method of the invention encompasses obtaining a previously selected sperm sample, as well as subjecting a source sample to any suitable selection method. Sperm from any species can be selected and frozen according to the method of the invention. The method can be carried out with sperm from domesticated animals, especially livestock, as well as with sperm from wild animals (e.g., endangered species). Preferably, the selected sperm sample contains mammalian sperm. Human sperm, bovine, equine, porcine, ovine, elk, and bison sperm are particularly preferred.

Generally, the selected sperm sample contains normal, viable sperm. To this end, the ejaculate from which the sperm are obtained typically has at least about 50%, and preferably at least about 75% morphologically normal sperm. In these embodiments, generally at least about 40%, and preferably at least about 60% of the sperm in the ejaculate exhibit progressive motility.

A wide variety of methods for selecting cells from a mixed populations are available, including, for example, selection based on binding of cells or cell components with antibodies, antibody fragments, or other binding partners and differential staining.

The invention is exemplified herein with selection based on sex-type, and sex-selected sperm for use in the invention can be obtained using any selection strategy that takes advantage of slight differences in characteristics between X- and Y-type sperm. Exemplary sex-selection methods include magnetic techniques (see, e.g., U.S. Patent No. 4,276,139), columnar techniques (see, e.g., U.S. Patent No. 5,514,537) gravimetric techniques (see, e.g., U.S. Patent No. 3,894,529, reissue Patent No. 32350, U.S. Patent Nos. 4,092,229, 4,067,965, and 4,155,831). Sex-selection based on differences in electrical properties is disclosed in U.S. Patent

5

No. 4,083,957, and techniques that select based on differences in electrical and gravimetric properties are discussed in U.S. Patent Nos. 4,225,405, 4,698,142, and 4,749,458. U.S. Patent Nos. 4,009,260 and 4,339,434 describe selection based on differences in motility. Biochemical techniques relying on antibodies are disclosed in U.S. Patent Nos. 4,511,661, 4,999,283,

5   3,687,803, 4,191,749, 4,448,767, whereas U.S. Patent Nos. 5,021,244, 5,346,990, 5,439,362, and 5,660,997 describe selection based on differences in membrane proteins.

Flow cytometry is a preferred method for separating cells from mixed populations based on differential staining with fluorescent dyes or binding to fluorescently labeled molecules, such as antibodies or nucleic acids. In fluorescence activated cell sorting ("FACS"), cells are "sorted"

10   into different populations based on the fluorescence intensity upon irradiation. FACS can be used for sex-selection of sperm because the X chromosome contains slightly more DNA than the Y chromosome. When sperm are stained with a fluorescent DNA-binding dye, X-chromosome bearing sperm absorb more dye than Y-chromosome bearing sperm and the two populations can therefore can be separated by FACS. This strategy was discussed in U.S. Patent No. 4,362,246

15   and significantly expanded upon in U.S. Patent No. 5,135,759 (issued to Johnson). Separation has been enhanced through the use of high-speed flow cytometers, such as the MoFlo® flow cytometer produced by Cytomation, Inc. (Ft. Collins, CO) and described in U.S. Patent Nos. 5,150,313, 5,602,039, 5,602,349, and 5,643,796, as well as in PCT Publication No. WO 96/12171.

20   The selection method used to obtain the selected sperm sample is preferably one that preserves sperm viability. Because of the relative fragility of sperm, normal flow cytometry techniques should generally be modified for sorting sperm. More specifically, the flow cytometry entails staining, dilution, and interrogation of cells with light. All of these steps represent stresses that can reduce sperm viability. The sensitivity of sperm to these stresses can

25   vary between species and even between individuals within species. Such sensitivities have either been documented or can readily be determined by empirical studies, such as those described in Examples 1-5.

Modifications that enhance viability are described the patent publications discussed above. For instance, procedures that provide improved sheath and collector systems for sorting

30   sperm are disclosed in PCT Publication No. WO 99/33956 (Application No. PCT/US98/27909). Further, Examples 1-7 below describe exemplary procedures for staining and sorting sperm.

6

Example 3 describes a study of the effects of laser intensity and dye concentration of post-thaw motility of sorted frozen sperm. This study indicates that the use of lower laser intensities during sorting can increase post-thaw motility.

The selected sperm sample can contain a variety of components besides sperm and will often contain components added to protect the sperm during the selection process. In the case of FACS, the selected sperm sample can contain component(s) of the solutions used for staining and sorting (e.g., the sheath fluid and the catch buffer).

In addition, the selected sperm sample typically contains an extender or extender fraction. For example, "two-step" extenders including an "A fraction" lacking glycerol and a "B fraction" containing glycerol are well known. The A fraction is added to sperm first, followed by addition of an equal volume of the B fraction. For this step, the B fraction is often divided into at least two aliquots and added sequentially; e.g., the second B fraction aliquot is added 15 minutes after the first.

If no extender components are present, an extender or extender fraction is typically added to the selected sperm sample before the sperm are isolated from the sample. If only some extender components are present, additional components can optionally be added so that selected sperm sample includes a complete extender or an extender fraction before the isolation step. In exemplary embodiments, bovine sperm are flow-sorted so as to produce a selected sperm sample including the A fraction of an extender (see Examples 2, 3, and 4). If desired, the B fraction can then be added to the selected sperm sample before the isolation step (see Example 5). The pre-isolation step extender (or extender fraction) is termed "the initial extender" to distinguish it from the "final extender" employed for the extension of isolated sperm before freezing. If the selected sperm sample was selected using FACS, the initial extender can be matched to the sheath fluid employed for sorting. Exemplary matched sheath fluids and extenders are described in detail in Example 4.

An extender suitable for use in the selected sperm sample includes a physiologically acceptable carrier. The physiologically acceptable carrier is typically aqueous, and, in preferred embodiments, includes deionized water. Suitable extenders commonly comprise one or more of the following additional components: a cryoprotectant, a component that maintains osmolality and buffers pH, an organic substance that prevents cold shock and preserves fertility of sperm, a detergent that acts synergistically with certain organic substances to enhance preservation of

7

sperm, an energy source that can be readily utilized by sperm, an antioxidant, which protects sperm from cold shock, a substance that facilitates sperm capacitation, and one or more antibiotics.

Although cryoprotectants useful in the invention are not limited to those acting by a particular mechanism, most conventional cryoprotectants act, at least in part, by reducing intracellular dehydration. Specifically, freezing is accompanied by an increase in solute concentration in the medium surrounding sperm. This increase in solute concentration draws water out of the cells, which increases intracellular electrolyte concentration. Exemplary cryoprotectants include glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and the like. The cryoprotectant suitable for use in a given extender can vary, depending on the species from which sperm are derived. For example, glycerol is suitable for use in cryopreservation of human and bovine sperm, but is generally not used for cryopreservation of porcine or rabbit sperm. Such preferences are well known for many commercially valuable sperm and can readily be determined empirically for other types of sperm.

The extender useful in the invention optionally includes one or more components that help maintain osmolality and provide buffering capacity. In preferred embodiments of the invention, the osmolality of the extender approximates that of physiological fluids. More preferably, the osmolality of the extender is in the range of about 280 mOsm to about 320 mOsm. The pH is also preferably within a physiologically acceptable range, more preferably in the range of about 6.5 to about 7.5.

Substances helpful in maintaining omolality and pH within these ranges are well known in the art and can be added to the extender as a solid or already in solution. A buffer containing a salt, a carbohydrate, or a combination thereof can be employed for this purpose. Specific examples include sodium citrate, Tris[hydroxymethyl]aminomethane, and TES (N-Tris [Hydroxymethyl]methyl-2-aminoethanesulfonic acid), and monosodium glutamate buffers; milk; HEPES-buffered medium; and any combination thereof. The component employed to help maintain osmolality and provide buffering capacity in a particular application can vary depending on the other components of the extender and, in some cases, on the species from which the sperm are derived. The selection of such a component for use in the present invention is, however, within the level of skill in the art.

8

One or more organic substances that protect sperm against cold shock and help preserve fertilizing capacity can also be included in the extender. Such substances are well known and are sometimes described as "nonpenetrating cryoprotectants." One skilled in the art can readily determine an organic substance suitable for a particular application of the cryopreservation

5   method described herein. For example, organic substances containing protective constituents (e.g., lipoproteins, phospholipids, lecithin) that are believed to reduce the impact of cold shock and the dilution effect can be included in the extender. Suitable organic substances include disaccharides, trisaccharides, and any combination thereof. Exemplary organic substances include egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, cholesterol,

10   and any combination thereof.

The extender can also include a detergent. Alkyl ionic detergents, such as sodium dodecyl sulfate (SDS), have been reported to act synergistically with egg yolk to enhance protection against cold shock. Other detergents useful in the cryopreservation of cells can also be employed in the extender, and the selection of a particular detergent for a specific application

15   is within the level of skill in the art in light of the guidance provided herein. See, e.g., Example 5.

Preferably, the extender includes an energy source that is readily utilized by sperm. In the absence of an energy source, sperm may oxidize intracellular phospholipids and other cellular components. Thus, the inclusion of an energy source in the extender protects intracellular

20   reserves and cellular components. As is well known in the art, sugars, particularly monosaccharides, provide a convenient energy source, although any conventional energy source can be employed in the extender. Exemplary monosaccharides useful in the extender include glucose, fructose, and/or mannose.

One or more antioxidants can optionally be included in the extender to provide additional

25   protection against cold shock. Exemplary antioxidants include butylated hydroxytoluene (BHT), its derivatives, and the like. However, other antioxidants useful in the cryopreservation of cells can also be employed in the extender, and the selection of a particular antioxidant for a specific application is within the level of skill in the art in light of the guidance provided herein.

The extender can also contain a substance that facilitates sperm capacitation. A variety of

30   capacitation facilitators are known in the art and any can be employed in the extender. Examples

9

include enzymes such as alpha amylase, beta amylase, beta glucuronidase, which can be used in combination, if desired.

Finally, the extender preferably includes an antibiotic, since substantial bacterial growth can threaten sperm viability and increase the risk of infection of the host in artificial insemination or in vitro fertilization procedures. Any of a variety of antibiotics useful in the cryopreservation of cells can also be employed in the extender. The selection of a suitable antibiotic depends on the species from which the sperm was obtained, the procedures involved in obtaining and handling the sperm sample, and the specific microorganism(s) to be targeted. Exemplary antibiotics include tylosin, gentamicin, lincomycin, spectinomycin, linco-spectin (a combination of lincomycin and spectinomycin), penicillin, streptomycin, and ticarcillin, which can be used alone or in combination. However, one skilled in the art can readily determine other antibiotics suitable for use in the extender.

Exemplary extenders are discussed in greater detail below and in the examples.

The sperm concentration is typically lower in the selected sperm sample than in the source sample, and, as indicated above, when FACS is employed, the dilution is significant. A typical sort based on sex-type can produce a sample containing sperm at 6×10^5 cells/ml catch buffer. As such a low concentration is not optimal for storage (at least for most species tested), the cryopreservation method of the invention generally concentrates the selected sperm sample.

Cooling the Selected Sperm Sample

The second step in the cryopreservation method entails cooling the selected sperm sample, typically, by reducing the temperature at a controlled rate. Cooling too rapidly can cause cold shock, which can result in a loss of membrane integrity and cell function. The severity of the effects of cold shock vary from species to species and depend on factors such as the rate of cooling and the temperature range. Under suitable controlled cooling conditions, the sperm are able to adapt to thermal effects. Example 2, among others, describes conditions for cooling bovine sperm, and determining suitable conditions for cooling sperm of other species is within the level of skill in the art.

In a preferred embodiment of the invention, the selected sperm sample is cooled typically from about 22°Celsius, to about 5°Celsius, and cooling is generally carried out over a period of about 60 minutes to about 24 hours, preferably over a period of about 90 minutes to about 240

10

minutes, and most preferably over a period of about 90 minutes to about 120 minutes. Cooling can be accomplished by any convenient method, including simply placing the selected sperm sample in a 5°Celsius environment.

5   *Isolation of Sperm Cells from the Selected Sperm Sample*

After initial extension of the selected sperm sample, sperm are isolated from the sample using any sufficiently gentle isolation method that provides at least about 50% recovery of sperm, more preferably about 75% to about 90% recovery of sperm, and most preferably about 80% to about 90% recovery of sperm. During the isolation step, the cooled sperm should

10   generally be kept cold, i.e., between about 1 and about 8°Celsius, and preferably close to 4 or 5°Celsius.

Any of a variety of methods suitable for recovering cells from a suspension can be used to isolate the sperm, including for example, filtration, sedimentation, and centrifugation. In an exemplary, preferred embodiment, the selected sperm sample is aliquoted into 50 ml tubes at

15   volumes not exceeding about 27 ml, and preferably between about 20 to about 27 ml. Centrifugation is carried out at about 4°Celsius, at about 850 x g, for about 20 minutes. Preferably, the centrifugation step provides at least about 50% to about 90% recovery of sperm, more preferably about 60% to about 90% recovery of sperm, and most preferably about 70% to about 90% recovery of sperm. After isolation, the supernatant is removed and the pellet is

20   suspended by vortexing gently or repeated aspiration at 4°Celsius. The sperm concentration is then typically determined (e.g., using a hemacytometer).

Final Extension of Isolated Sperm Cells

After isolation, the sperm are pooled, if desired, and extended with final extender to an

25   appropriate concentration for freezing. The concentration of sperm after the final extension and prior to freezing is preferably in the range of about 1×10^6/ml to about 300×10^6/ml, more preferably about 10×10^6/ml to about 50×10^6/ml, and most preferably about 10×10^6/ml to about 20×10^6/ml.

The description of the initial extender above also applies to the final extender, which can

30   be the same as or different from the initial extender. In particular embodiments, the composition

11

of the sperm sample extended with the final extender is substantially similar to (if not the same as) the composition of the sperm sample after addition of the initial extender.

In a preferred embodiment of the invention, an egg yolk-Tris extender is used. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); citric acid

5   and Tris[hydroxymethyl]aminomethane (buffer); egg yolk (organic substance); fructose (energy source); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

10

15

20

25

30

12

Components of Egg Yolk-Tris Extender

Glycerol:	4-8% vol/vol
Citric Acid:	55-75 mM
Tris [hydroxymethyl]aminomethane:	190-210 mM
Egg yolk:	5-25% vol/vol
Fructose:	45-65 mM
Tylosin:	25-100 µg/ml
Gentamicin:	200-300 µg/ml
Linco-spectin:	100-400 µg/ml[*]

[*] 100-400 µg/ml lincomycin and 100-400 µg/ml spectinomycin

In a variation of this embodiment particularly suitable for freezing bovine sperm, the concentrations of these components after addition of the final extender to the isolated sperm are about 6% (vol/vol) glycerol, about 65 mM citric acid, about 200 mM Tris[hydroxymethyl]aminomethane, about 20% (vol/vol) egg yolk, about 56 mM fructose, about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 150/300 µg/ml linco-spectin (i.e., 150 µg/ml lincomycin and 300 µg/ml spectinomycin), in deionized water.

In an alternative embodiment, an egg yolk-citrate extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); sodium citrate (buffer); egg yolk (organic substance); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

13

<u>Components of Egg Yolk-Citrate Extender</u>

Glycerol:	4-8% vol/vol
Sodium Citrate :	60-80 mM
Egg yolk:	5-25% vol/vol
Tylosin:	25-100 µg/ml
Gentamicin:	200-300 µg/ml
Linco-spectin:	100-400 µg/mL*

*100-400 µg/ml lincomycin and 100-400 µg/ml spectinomycin

Exemplary, preferred concentrations for freezing bovine sperm are about 7% (vol/vol) glycerol, about 72 mM sodium citrate, about 20% (vol/vol) egg yolk, about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 250/300 µg/ml linco-spectin.

In another alternative embodiment, an egg yolk-TES-Tris ("EY TEST") extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); egg yolk and heated milk, e.g., homogenized milk containing 1.25% fructose with 10% glycerol (organic substances); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

14

Components of Egg Yolk TES-Tris Extender

Glycerol:	3-7% vol/vol
Tris [hydroxymethy-methyl]-2-aminoethanesulfonic acid:	140-170 mM
Tris [hydroxymethyl]aminomethane:	60-80 mM
Egg yolk:	5-25% vol/vol
Fructose:	5-12 mM
Tylosin:	50-150 µg/ml
Gentamicin:	400-600 µg/ml
Linco-spectin:	200-700 µg/mL*

5

10

*200-700 µg/ml lincomycin and 200-700 µg/ml spectinomycin

Exemplary, preferred concentrations for freezing bovine sperm are about 5% (vol/vol) glycerol, about 158mM Tris[hydroxymethy-methyl]-2-aminoethanesulfonic acid, about 72 mM

15 Tris[hydroxymethyl]aminomethane, about 20% (vol/vol) egg yolk, about 8mM fructose, about 100 µg/ml tylosin, about 500 µg/ml gentamicin, and about 300/600 µg/ml linco-spectin.

In yet another alternative embodiment of the invention, a Milk extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); heated homogenized milk (organic substance); fructose (energy source); and tylosin, gentamicin,

20 and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

25

30

15

<div align="center">Components of Milk Extender</div>

Homogenized Milk	90% (vol/vol)
Glycerol:	3-7% (vol/vol)
5 Fructose:	1.25 % (wt/vol)
Tylosin:	50 μg/ml
Gentamicin:	250 μg/ml
Linco-spectin:	250/300 μg/ml

*

10   * 250-300 μg/ml lincomycin and 250-300 μg/ml spectinomycin

Exemplary preferred concentrations for freezing bovine sperm are about 90% milk, about 10% (vol/vol) glycerol, about 1.25% fructose (wt/vol?), about 50 μg/ml tylosin, about 250 μg/ml gentamicin, and about 250/300 μg/ml linco-spectin.

15   Other extenders standardly used to freeze sperm can also be employed as the final extender in freezing selected sperm. A variety of extenders optimized for use in freezing sperm from various species have been described, and many are commercially available. Freezing extenders for equine sperm typically consist of milk, egg yolk, various sugars, electrolytes and a cryoprotectant. Exemplary freezing extenders are described by Squires, E.L., et al., Cooled and

20   Frozen Stallion Semen Animal Reprod. and Biotechnology Laboratory, Bulletin No. 69, Chapter 8, "Seminal Extenders" pp. 49-51 (July, 1999).

Equilibration and Freezing of Sperm

Extension of the sperm sample produces a suspension of sperm, which is then transferred 25   into containers for freezing. If the sperm are intended for use in fertilization, the cells are conveniently aliquoted into individual doses sufficient to achieve fertilization. The required dose can vary substantially from one species to the next and is either well-known (e.g., for cattle and horses) or can readily be determined. In the case of sex-selected bovine sperm, convenient doses range from about 1.0×10^6 sperm to about 3.0×10^6 sperm.

30   Any suitable container can be employed for freezing, including, for example, an ampule, a vial, and a straw. Sperm intended for AI are typically frozen in straws (e.g., 0.25 ml or 0.50 ml

<div align="center">16</div>

straws) designed for use with an insemination gun. Preferably, a bolus of extender is drawn into the straw, followed, in sequence, by air, sperm, air, and extender, so that the sperm are flanked on either side by an air space, which separates the sperm from a bolus of extender at either end of the straw.

5   Prior to freezing, the sperm are generally allowed to equilibrate at about 5°C. Preferably, the sperm are allowed to equilibrate for a period in the range of about 1 hour to about 18 hours, more preferably between about 3 hours and about 18 hours, and most preferably between about 3 hours and about 6 hours (see Example 2).

       Following equilibration, any standard freezing method can be employed, provided the

10 freezing rate is not too rapid (i.e., not in excess of about 0.5°C/minute). Preferably, the freezing rate is about 0.5°C/minute. In an exemplary, preferred embodiment, the sperm are placed in static liquid nitrogen vapor, and freezing is carried out in three distinct stages over a period of about 10 minutes. In the first stage of freezing, the sperm are cooled from about 5°C to about $^-15$°C at a rate of about 40°C/minute to about 65°C/minute. In the second stage of freezing, the

15 sperm are cooled from about $^-15$°C to about $^-60$°C at a rate of about 25°C/minute to about 35°C/minute. In the third stage, the sperm are plunged into liquid nitrogen at about $^-100$°C.

Selected Sperm Samples

       In addition to a freezing method, the invention provides a frozen sperm sample including

20 sperm selected from a source sample for a particular characteristic. The sperm can be from any species, including any of those discussed above with reference to the freezing method. The invention encompasses frozen sperm selected for any characteristic by any suitable method, such as those described above. Preferred embodiments include frozen sex-selected human, bovine, equine, porcine, ovine, elk, or bison sperm. Sex-selection is preferably carried out using flow

25 cytometry as described generally above.

       Also within the scope of the invention is a container containing a frozen sperm sample according to the invention. The container can be formed from any material that does not react with the frozen sperm sample and can have any shape or other feature that facilitates use of the sample for the intended application. For samples intended for use in AI, for example, the

30 container is conveniently a straw (e.g., 0.25 ml or 0.5 ml straw) designed for use with an insemination gun. The container is sealed in any manner suitable for preserving the sample at

17

the intended storage temperature, which is typically below -80°Celsius. 0.25 ml straws can be sealed, for instance, with PVC powder, ultrasonically, or with a cotton-polyvinyl plug and/or a stainless steel ball (BB).

As the frozen sperm sample of the invention is typically thawed before use, the invention also provides a thawed, previously frozen, selected sperm sample and a container including such a thawed sample.

Methods of Using the Selected Sperm Sample

The frozen selected sperm sample of the invention is suitable for use in any method in which sperm are used. The sample can be thawed and used in any conventional fertilization method, such as artificial insemination or in vitro fertilization. Thawing is carried out in the same manner as for frozen, non-selected sperm. Briefly, the straw containing the frozen sperm is submerged in a water bath maintained at a temperature of about 35°C to about 37°C for a period of about 20 to about 30 seconds. After thawing, semen deposition (e.g., insemination) is carried out according to standard procedures, taking care to protect the sperm from environmental fluctuations.

18

EXAMPLES

Example 1

Effects Of Dilution On Sperm

5   Objective: to determine the effect of sperm concentration on sperm motility for non-frozen, non-sorted, but highly diluted sperm.

A.   *Effects of Dilution on Non-washed Sperm*

1.   *Collection of Source Sample.* Sperm were collected from bulls on a routine

10   collection schedule using an artificial vagina as described in Schenk J., Proc 17th NAAB, p.48-58 (1998), and Saacke RG, Proc NAAB Tech Conf AI Reprod. 41:22-27 (1972). All ejaculates used contained greater than 50% progressively motile and greater than 75% morphologically normal sperm. Antibiotics were added to the raw ejaculate as described by Shin S., Proc NAAB Tech Conf AI Reprod. 11:33-38 (1986) within 15 minutes of collection, and the concentration of

15   sperm was determined using a spectrophotometer.

2.   *Methods.* Sperm from 4 bulls were diluted to 1.25, 2.5, 5, 10, 15, and 20 × 10^6/ml using an egg yolk-citrate extender (EYC) prepared with 20% egg yolk (vol/vol) in 72 mM sodium citrate, 50 µg/ml tylosin, 250 µg/ml gentamicin, and 250/300 µg/ml linco-spectin. Each

20   sample was prepared in duplicate (2 tubes/dilution/bull) and comprised 8 ml total volume per tube. All samples were incubated for 60 minutes at 22°C, after which they were centrifuged using a swinging bucket centrifuge (Eppendorf, Model # 5810R) at 600 × g for 10 minutes to concentrate the sperm. After centrifugation, the supernatant from one set of the duplicate tubes was not removed; the sperm were resuspended in the same medium and at the original

25   concentration by repeated gentle aspiration using a 5-ml serological pipette. (The second set of the duplicate tubes were used in Example 1B.) Sperm samples were then cooled to 5°C at 0.2°C/min over 90 minutes. These sperm were termed "non-washed sperm." All samples were incubated at 5°C for 24 or 48 h post-collection.

30   3.   *Evaluation of Motility.* After incubation, the samples were warmed to 37°C using a dry block incubator for 10 minutes prior to determination of motility. For this experiment, a

19

single, blind estimate of the percentage of progressively motile sperm was determined for each sample. Progressive sperm motility was determined subjectively for each subclass by a single observer (x200, phase-contrast microscopy); another person prepared the microscope slides in a randomized manner so the observer was unaware of treatments.

5

4.   *Statistical Analysis*. Data were analyzed by analysis of variance (SAS Institute, Cary, North Carolina) with factors bulls and initial dilution concentration. Separate analyses were done for each incubation time. Dilution trends were tested using (log) linear contrasts.

5.   *Results*. Data for non-washed sperm (Table 1) revealed (log) linear relationships

10   ($P<0.01$) for both incubation times. Percentages of motile sperm increased as sperm concentration increased from 1.25×10^{6}/ml to 10×10^{6}/ml, but there was little difference thereafter. The cubic term was significant ($P<0.05$) for 24-h and marginally significant ($P<0.1$) for 48-h incubations. There was a bull effect ($P<0.01$) at both times.

15

20

25

30

Table 1. Effects of cooling on non-washed sperm
motility (%) after cooling to 5°C.

Dilution (10^6/ml)	Incubation at 5°C	
	24 h[a]	48 h[b]
1.25	18[c]	0[c]
2.5	38[c,d]	6[c,d]
5.0	56[d]	31[d,e]
10.0	61[d]	42[e]
15.0	55[d]	44[e]
20.0	58[d]	41[e]
S.E.[f]	5.6	6.4

[a] (log) linear ($P<0.01$) and cubic effects ($P<0.05$).

[b] (log) linear ($P<0.01$) and cubic effects ($P<0.1$).

[c,d,e] Means within columns without common
superscripts differ ($P<0.05$).

[f] $\sqrt{error\ mean\ square\ of\ ANOVA} \div \sqrt{N}$
(SAS Institute, Cary, NC, USA)

B.   *Effects of Dilution on Washed Sperm*

1.   *Collection of Source Sample*. The second set of the duplicate tubes containing samples prepared in Example 1A were used in this experiment.

2.   *Methods*. The sperm were diluted, incubated and concentrated by centrifugation as in Example 1A. Following centrifugation, 7.1 ml of the supernatant was aspirated from each tube, removing most of the seminal plasma and leaving the sperm in a 900-μl pellet. The sperm were diluted with EYC (see Example 1A) to make 10×10^6/ml or 20×10^6/ml sperm suspensions. The samples were then cooled to 5°C over 90 minutes as in Example 1A.

3.   *Evaluation of Motility*. The samples were warmed and evaluated for progressive motility as in Example 1A.

21

4.	_Statistical Analysis_. Data were analyzed as in Example 1A. In addition, data in Example 1B were analyzed for incubation concentration at 5°C.

5.	_Results_. Data for washed sperm (Table 2) revealed no significant treatment effects when sperm were evaluated after 24 h. However, after storage for 48 h at 5°C, there were bull, initial dilution, incubation concentration and bull by incubation effects ($P<0.05$). More sperm remained motile when held at 20×10^6 /ml than at 10×10^6 /ml (31% vs. 20%; $P<0.05$). Initial dilutions of 1.25, 2.5, and 5×10^6 sperm/ml resulted in lower progressive motility than 10×10^6 sperm/ml ($P<0.05$), with respective main effect means of 19, 20, 27, and 37% motile sperm.

Table 2. Cumulative effects of washing, dilution, concentration and cooling on progressive sperm motility (%)

Sperm conc (10^6/ml) during 1 h preincubation at 37°C	Storage at 5°C – Sperm Concentration and Duration			
	24 h		48 h[a]	
	20×10^6/ml	10×10^6/ml	20×10^6/ml	10×10^6/ml[b]
1.25	45	49	24	15
2.5	51	40	29	11
5.0	54	54	32	21
10.0	51	50	40	34
15.0	60		41	
20.0	55		40	

[a] Concentration to 20×10^6 sperm/ml was superior ($P<0.05$) to 10×10^6 sperm/ml after 48 h storage.
Also, initial dilution to 10×10^6 was superior to lower dilutions ($P<0.05$).
Pooled standard errors ($\sqrt{error\ mean\ square\ of\ ANOVA} \div \sqrt{N}$ were 4.0 for 24 h, and 2.8 for 48 h incubations.

[b] Significant (log) linear trend ($P<0.06$).

C.	_Conclusion_

High sperm dilution and cooling resulted in a substantial reduction in the percentage of motile sperm, regardless of the presence or removal of seminal plasma. However, this dilution

22

effect was greatly attenuated by concentrating the diluted sperm to 10×10^6/ml and even more, to 20×10^6/ml before storage at 5°C. Sperm from some bulls tolerated dilution better than sperm from other bulls; however, the bull differences found are typical. Extremely dilute sperm might be compromised during sorting, in part, by removal of protective compounds in seminal plasma.

5

Example 2

Effects of Equilibration Time Before Freezing Sorted Sperm

Objective: to evaluate the effect of equilibration times (3, 6 and 18 h, 5°C) before freezing on flow-sorted sperm.

10

The following experiment was replicated in its entirety using the same bulls:

1.   *Collection of Source Sample*. Sperm of 4 bulls were collected and prepared as described in Example 1A.

2.   *Methods*.

15

a)   *Staining and Preparation for Sort.*

i)   Preparation of Stain Stock Solution: a stock solution of 8.89 mM Hoechst 33342 (bis-Benzimide H-33342; #190305, ICN Biomedicals Inc., Aurora, OH) was prepared in deionized water.

20

ii)   Sperm Stain Procedure: sperm were diluted in a modified TALP buffer (Table 3) to 400×10^6 sperm/ml. Following dilution, Hoechst 33342 dye was added to the sperm suspensions at a concentration of 224 µM. After the stain was added to the sperm suspensions, the samples were incubated for 60 minutes at 34°C. Following incubation, sperm were diluted to 100×10^6/ml with TALP containing 2.67% clarified egg yolk and 0.002% food coloring dye (FD&C #40) which quenches the fluorescence of Hoechst 33342 in sperm with compromised cell membranes, allowing them to be gated out during the sorting process. Just prior to flow sorting, samples were filtered at unit gravity through a 40-µm nylon mesh filter to remove any debris and/or clumped sperm.

25

30

23

b)   _Sorting_. A two-line argon laser operating at 351 and 364 nm and 150 mW was used to excite the Hoechst 33342 dye. The flow cytometer/cell sorter used was an SX MoFlo® (Cytomation, Inc., Fort Collins, CO, USA) operating at 50 psi. A Tris-based sheath fluid was used, consisting of Tris (hydroxymethyl) aminomethane (Tris; 197.0 mM; #T-1503, Sigma Chemical Co., St. Louis, MO, USA), citric acid monohydrate (55.4 mM; #C-7129, Sigma Chemical Co., St. Louis, MO, USA) and fructose (47.5 mM; #F-0127, Sigma Chemical Co., St. Louis, MO, USA). Baseline antibiotics were also added to the Tris-based sheath fluid consisting of 0.58 g/L of penicillin and 0.05 g/L of streptomycin sulfate.

The sperm were sorted by a process referred to as "bulk sorting" which permits rapid accumulation of large numbers of sperm so that large-scale examples can be done within a reasonable time. The sperm pass through the flow cytometer under the standard operating conditions with the exception that all droplets containing viable sperm were collected into a single tube rather than being sorted into 2 tubes based upon specific characteristics (e.g., sorting by sex-type). Sperm were sorted on the basis of viability; hence, sperm that have compromised plasma membranes were excluded during bulk sorting.

Stained sperm were maintained at 22 ± 1°C during sorting. Bulk sorted sperm were collected in 50-ml plastic tubes containing 2 ml of 20% egg yolk-Tris extender prepared with 20% egg yolk (vol/vol) in 200 mM Tris, 65 mM citric acid, 56 mM fructose, 50 μg/ml tylosin, 250 μg/ml gentamicin, and 150/300 μg/ml linco-spectin in deionized water. The egg yolk-Tris extender was termed "Tris-A fraction" to denote the lack of glycerol at this point in the procedure. Sperm were collected in tubes to contain 12 ml and approximately 6×10^6 sperm. The sperm were subsequently incubated at 22°C for 1 to 3 h to simulate conditions of a sort based on sex-type.

c)   _Preparation for Freezing._ Following incubation, the sorted sperm were cooled to 5°C over the period of 70 minutes. After cooling, the contents of the two tubes were pooled and transferred to a refrigerated, swinging bucket centrifuge set at 5°C and

24

centrifuged at 850 × g for 20 minutes. After removing the supernatant, processing continued at 5°C by adding about 150 μl of Tris-A fraction extender to about 150-μl of sperm pellet to bring the sperm concentration to approximately 40 × 10⁶/ml. The sperm of individual bulls were pooled and diluted immediately with an equal volume of egg yolk-Tris extender containing 12% (v/v) glycerol ("Tris-B fraction"). The Tris-B fraction was added to the sperm suspension as 2 equal volumes at 15-minute intervals to adjust the final sperm concentration to 20×10^6/ml. The final glycerol concentration of the complete egg yolk-Tris extender containing the sperm was 6% (v/v).

d)   *Equilibration and Freezing*. Extended sperm were then packaged into 0.25-ml polyvinylchloride straws to be frozen by routine procedures on racks in static liquid nitrogen vapor. Two straws from each of 4 bulls were frozen after 3, 6 and 18 h of total equilibration time at 5°C.

3.   *Evaluation of Post-Thaw Motility*. Straws were thawed in a 37°C water bath for 30 sec. Blind estimates of progressive motility were made after incubating samples at 37°C for 0, 1 and 2 h post-thawing. Each of two observers estimated progressive sperm motility from each of two straws of semen. These four blind estimates for each experimental unit represent subsampling.

4.   *Statistical Analysis*. Statistically, the subsamples were analyzed as a subplot to the main plot least-squares ANOVAs to analyze effects of any observer and observer x treatment interaction. N refers to the number of experimental units, not subsamples; standard errors were calculated on the basis of means of the 4 subsamples from error mean squares of ANOVAs and the numbers of experimental units; least-squares means are presented.

Treatment effects were evaluated via separate ANOVAs for each incubation time. The model included bulls as a random effect and equilibration time and observer as fixed effects; the subplot consisted of the observer term and related interactions.

25

5.   *Results*. The 3- or 6-h equilibration times were superior to 18-h (Table 4), based on the percentage of progressively motile sperm, for 0 and 1 h ($P<0.01$) but not 2 h of post-thaw incubation. Effects of bull were evident at 1 and 2 h incubation times ($P<0.05$), but not at 0 h. There was no significant ($P>0.1$) bull by equilibration time interaction nor was there a significant observer effect for any response.

Table 3. Modified TALP Buffer

Ingredient	Concentration
NaCl	95.0 mM
KCl	3.0 mM
$NaHPO_4$	0.3 mM
$NaHCO_3$	10.0 mM
$MgCl_2 \bullet 6H_2O$	0.4 mM
Na Pyruvate	2.0 mM
Glucose	5.0 mM
Na lactate	25.0 mM
HEPES[a]	40.0 mM
Bovine serum albumin[b]	3.0 mg/ml
Gentamycin Sulfate	30.0 μg/ml

[a] #H3375, Sigma Chemical Co., St. Louis, MO, USA

[b] #US70195, fraction V; Amersham/Life Science, Cleveland, OH, USA

26

Table 4. Effect of pre-freeze equilibration time on post-thaw progressive motility (%)

Equilibration at 5°C	Post-thaw incubation at 37°C		
	0 h	1 h	2 h
3 h	41[a]	36[a,b]	16
6 h	41[a]	37[a]	18
18 h	35[b]	31[b]	12
S.E.[c]	1.5	0.8	2.0

[a,b] Within columns, means without common superscripts differ (P<0.05), Tukey's HSD.

[c] Pooled standard errors,

$$\sqrt{error\ mean\ square\ of\ ANOVA} \div \sqrt{N}$$

6.   _Conclusion_. The results indicated no differences in post-thaw sperm motility between 3 and 6 h of total equilibration time at 5°C, but there was a significant decline in sperm motility following 18 h of equilibration at 5°C before freezing. The 3- to 6-h range permits pooling 2 consecutive 3-h sorting batches for freezing sperm without decreasing post-thaw motility.

As the bull by equilibration-time interaction was not significant, 3 to 6 h equilibration was adequate, with the caveat that only 4 bulls were used. The optimum equilibration time for a minority of bulls is expected to be >6 h.

27

Example 3

Effects Of Stain Concentration And Laser Power on Sorted Sperm

Objective: to evaluate the effects of Hoechst 33342 dye concentration in combination with laser intensity on flow-sorted sperm.

5

1.   _Collection of Source Sample_. Sperm of 6 bulls were collected and prepared as described in Example 1A.

2.   _Methods_.

10

a)   _Experimental Design_. One ejaculate (2 bulls) and 2 ejaculates on different days (4 bulls) were used in a 2 by 2 design plus control.

b)   _Staining and Sorting_. Staining, preparation for sorting and sorting sperm were achieved as described in Example 2 except that the Hoechst 33342 dye was added to sperm suspensions at a final concentration of 149 μM or 224 μM; and sperm were

15 bulked-sorted with the laser operating at 100 mW or 150 mW of incident power. Bulk-sorted sperm were collected into 50-ml plastic tubes as described in Example 2. Four tubes containing approximately 15×10^6 total sperm/tube were collected over 1 h for each bull. The sorted sperm were incubated for 1 h at 22°C to simulate a longer sorting time.

20

c)   _Preparation for Freezing_. Following incubation, the sperm were cooled as in Example 2. The sperm were then concentrated by centrifugation at 5°C at $850 \times g$ for 20 minutes. After removing the supernatant, 150 μl of Tris-A fraction extender was added to each 150-μl sperm pellet at 5°C. All of the sperm pellets were suspended by

25 gentle repeated aspiration and the sperm of individual bulls were pooled. Tris B-fraction extender was added stepwise as described in Example 2. A non-stained, non-sorted control for each bull was prepared at 20×10^6 sperm/ml in Tris extender containing 6% glycerol and cooled to 5°C while the bulk-sorted sperm were being prepared.

30

28

52011067.1/22091-701

d)   *Equilibration and Freezing*. The control and sorted sperm were packaged into 0.25-ml polyvinylchloride straws as described in Example 2, equilibrated at 5°C for 3 h and then frozen conventionally.

5   3.   *Evaluation of Post-Thaw Motility*. Straws were thawed and evaluated as described in Example 2.

4.   *Statistical Analysis*. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via ANOVA. The model included dye

10   concentration, laser intensity and bulls in the main plot, and observer and related interactions in the subplot. Bulls were considered a random effect and the other factors as fixed.

5.   *Results*. Bull effects were significant for percentages of progressively motile sperm immediately after thawing ($P<0.1$) and after 1 h and 2 h of incubation at 37°C ($P<0.05$).

15   There was no effect of dye concentration or bull by dye concentration on sperm motility at any incubation time. With bulls considered as a random effect, 150 mW of laser power resulted in lower post-thaw motility of sperm than 100 mW at 0 h of incubation ($P<0.1$), but not at other incubation times (Table 5). If bulls are considered as fixed effects, 150 mW of power resulted in lower sperm motility than 100 mW ($P<0.05$) at all 3 incubation times. There was an effect of

20   bull by laser power ($P<0.05$) on sperm motility at 1 h, but not at 0-h or 2-h incubation times. Also, the higher laser power resulted in lower sperm motility than the control ($P<0.05$) at 0- and 1-h incubation times (Table 5). There was a significant observer effect at 1-h, but not at 0=h or 2-h, incubation times. There was no observer by treatment interaction ($P>0.1$).

25

30

29

Table 5. Effects of laser intensity and dye concentration on post-thaw motility (%).

Main effect means	Incubation at 37°C		
	0 h	1 h	2 h
Control	49	44	33
Dye Concentration			
149 µM	41	39	30
224 µM	42	39	30
Laser Intensity			
100 mW	46	42	33
150 mW	38[a]	35[b]	27
S.E.[c]	2.2	1.2	1.3

[a] Significant main effect ($P<0.1$) and differs from control ($P<0.05$).

[b] Differs from control ($P<0.05$).

[c] Pooled standard errors, $\sqrt{error\ mean\ square\ of\ ANOVA} \div \sqrt{N}$

6. *Conclusion*. Percentages of progressively motile sperm post-thaw were diminished by the staining and sorting process. Higher laser intensity was more damaging than the lower laser intensity. There was no effect of dye concentration on post-thaw sperm motility. Thus, excitation of the sperm-bound Hoechst 33342 dye at lower laser intensities is less damaging and that staining sperm at the higher dye concentration had no detrimental effect on post-thaw motility. The damage observed was presumably to the sperm-motility apparatus.

30

Example 4

Evaluation of Pre-Sort Staining Procedures and
Selection of Extenders for the Cryopreservation Of Sperm

Objective: (1) to evaluate three pre-sort treatments for sperm; and, (2) and to evaluate sheath fluid and extender combinations for the cryopreservation of flow-sorted sperm.

The following experiment was replicated in its entirety:

1. _Collection of Source Sample_. Sperm from 4 bulls were collected and prepared as described in Example 1A.

2. _Methods_.

a) _Experimental Design_. A 3 (pre-sort treatments) by 3 (extenders) by 2 (sheath fluids) by 4 (bulls) by 2 (observers) factorial experiment was designed to determine the best procedure to hold sperm prior to sorting, and to evaluate three extenders for cryopreserving the sorted sperm. .

b) _Sample Preparation and Staining_. Freshly collected sperm from each of 4 bulls were treated as follows:

(1) diluted to 400×10^6 /ml in modified TALP (see Example 2, Table 3) and stained for 1 h at 34°C before bulk-sorting ("Dilute – 0 h");

(2) incubated neat at 22°C for 3 h before dilution, staining and sorting ("Neat – 3 h"); or,

(3) diluted and stained as "Dilute-0 h" and then incubated at 22°C for 3 h before bulk-sorting ("Diluted – 3 h").

c) _Extenders_. The following freezing extenders were compared: EYC (see Example 1) containing 7% glycerol, egg yolk-Tris (see Example 2) containing 6% glycerol, and egg yolk-TES-Tris (TEST) containing 5% glycerol. EYC "A Fraction" refers to the EYC extender containing no glycerol, and EYC "B Fraction" refers to EYC extender containing twice the final, desired glycerol

31

concentration (i.e., 14%). Thus, when EYC A and B fractions are combined in equal volume, the final EYC extender contains 7% glycerol. Tris A and B fractions are similarly named, and described in Example 2. TEST extender is prepared as a complete extender containing 5% glycerol; hence, there were no "A" and "B" fractions for TEST.

d)   *Sheath Fluid*. Sheath fluid was either 98.6 mM sodium citrate dihydrate (#S279-3, Fisher Scientific, Fair Lawn, NJ) or Tris as described in Example 2. Both types of sheath fluid were adjusted to pH 6.8; osmolality was about 270 to 280 mOsm/kg. Tris sheath fluid was used to collect sperm that were later extended in egg yolk-Tris and TEST freezing extenders. Sheath fluid containing 98.6 mM sodium citrate dihydrate was used to collect sperm to later be extended in EYC freezing extender.

e)   *Sorting*. Approximately 58×10^6 sperm for each combination of pre-sort treatment, sheath fluid and extender were bulk-sorted as described in Example 2 using 150 mW of incident laser power. For each sort, sperm were collected over approximately 1 h. After sorting, the samples were incubated at 22°C for 2 h to simulate a 3 h sort.

f)   *Preparation for Freezing*. Following incubation, the sperm were cooled as described in Example 2. After cooling, the samples were centrifuged at 5°C at $850 \times g$ for 20 min. Each sample comprised about 28 ml total volume and was contained in a 50-ml plastic tube

After the supernatant was removed, the sperm were returned to a 5°C cold room for extension. Samples were extended to 40×10^6/ml by depositing 131 µl of the sperm suspension into 69 µl of A-fraction EYC, A-fraction egg yolk-Tris, or TEST extender. Immediately, suspensions were adjusted to 20×10^6 sperm/ml with the addition of the matched glycerol containing extender (i.e., B-fraction EYC, B-fraction Tris) or TEST. B-fraction extenders were added to their respective samples stepwise (2X) at 15-min intervals as described in Example 2.

32

The TEST was added to sperm stepwise in the same manner as B-fraction EYC and Tris extenders.

g)   *Equilibration and Freezing*. Sperm were packaged into 0.25-ml polyvinylchloride straws, equilibrated for 3 h at 5°C and then frozen in static liquid nitrogen vapor.

3.   *Evaluation of Post-Thaw Motility*. Thawing and post-thaw evaluations of sperm were done as described for Example 2.

4.   *Statistical Analysis*. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via separate analyses of variance for each post-thaw incubation time. The main plot included pre-sort treatment, extenders, and bulls; the subplot consisted of observers and associated interactions. Bulls were considered a random effect, and the other factors, fixed. The entire experiment was replicated twice. Tukey's HSD test was used to separate means.

5.   *Results*. Post-thaw progressive motility of bulk-sorted sperm was affected (P<0.05) by extender and bulls at each post-thaw incubation time and by pre-sort procedure at 0 h of incubation (Table 6). There were no differences due to sheath fluids (P>0.05). At 0-h post-thaw incubation, use of the neat-3h treatment resulted in more motile sperm after freezing and thawing than the other 2 pre-sort staining treatments (P<0.05; Table 6). However, pre-sort procedures were not statistically significant after post-thaw incubation of sperm for 1 or 2 h with bulls considered as a random effect. Importantly, at these 2 incubation times, there were significant pre-sort treatment by bull interactions (P<0.05). Furthermore, pre-sort treatment would have been a significant effect at all post-thaw incubation times had bulls been considered as fixed effects.

Immediately after thawing (0 h), TEST was the best extender, but after 1 or 2 h of incubation of 37°C, Tris was the best extender. Importantly, there was no pre-sort treatment by extender interaction for any response. There were observer effects (P<0.01) at all incubation

33

times, but no observer by treatment interactions. There was a bull by extender interaction (P<0.05) at all 3 incubation times.

Table 6. Main effects of pre-sort treatment and freezing extenders on post-thaw progressive motility (%)

Pre-sort procedure	Extender	Incubation at 37°C		
		0 h	1 h	2 h
Dilute – 0 h	Mean	39[a]	32	22
Neat – 3 h	Mean	43[b]	36	25
Dilute – 3 h	Mean	38[a]	31	19
Mean	EYC	36[a]	29[a]	17[a]
Mean	Tris	40[b]	39[b]	29[b]
Mean	TEST	44[c]	33[c]	20[a]
S.E.[d]		0.8	0.8	0.7

[a,b,c] Means within columns, within main effects, without common superscripts differ (P<0.05).

[d] Pooled standard errors $= \sqrt{error\ mean\ square\ of\ ANOVA} \div \sqrt{N}$

6. _Conclusion_. This study showed that holding sperm neat for 3 h before dilution, staining and sorting was better than immediate dilution and staining 0 h or 3 h later. Thus, by 3 h into the sort, it is best to continue with a new aliquot of the original ejaculate that was held neat 3 h and then stained, rather than continuing with the original sample of sperm stained and held at 400×10^6 sperm/ml.

Even though TEST extender provided higher post-thaw motility at 0 h, Tris was the superior extender when sperm were stressed by incubation at 37°C. Either sheath fluid worked equally well for each extender. Based on these results, we have incorporated the use of Tris sheath fluid in combination with Tris freezing extender into our standard operating procedure.

<u>Example 5</u>

Effects Of Extender Additives on Sorted Sperm

Objective: to evaluate the effect of adding sodium dodecyl sulfate ("SDS") to the freezing extender on flow-sorted sperm.

5

A. *Evaluation of Effect of Concentration of SDS in Freezing Extender*

1. <u>*Collection of Source Sample*</u>. Sperm of 6 bulls were collected and prepared as described in Example 1A.

10 2. <u>*Methods*</u>. Sperm from each of 6 bulls were extended to 20×10^6/ml in 20% whole egg Tris ("WET") extender containing 0, 0.03, 0.06, 0.09, or 0.12 percent SDS, packaged into straws and frozen. WET extender was prepared using 3.028 g of Tris[hydroxymethyl] aminomethane, 1.78 g of citric acid monohydrate, and 1.25 g of fructose per 100 ml of double distilled water, to which 20% whole egg (vol/vol) was added. The WET extender was prepared
15 at a pH of about 7.0 and contained a final glycerol concentration of about 6% (vol/vol). The WET extender also contained 1000 IU of penicillin "G" sodium and 100 μg of streptomycin sulfate/ml.

3. <u>*Results*</u>. The respective means (n = 1 sample from each of 6 bulls) were 51, 51,
20 50, 51, and 48% progressive motile sperm approximately 10 minutes post-thaw. Based on these results, 0.06 percent SDS was used in Example 5B.

B. *Evaluation of the Effects of 0.06 Percent SDS in Various Freezing Extenders on Post-Thaw Motility of Flow-Sorted Sperm*

25 1. <u>*Collection of Source Sample*</u>. Sperm of 8 bulls were collected and prepared as described in Example 1A.

2. <u>*Methods*</u>. Post-thaw motility was studied for sperm frozen in egg yolk-Tris (see Example 2) and WET extenders (see Example 5A) with and without 0.06% SDS. Final glycerol content for both extenders was 6%.
30

35

a)   *Staining, Preparation for Sort, Sorting*. Stained sperm samples were prepared from an ejaculate from each of 8 bulls as described in Example 2. Stained sperm were bulked-sorted using Tris sheath fluid as described in Example 2 except that the sort was achieved using 135 mW of incident laser power. Sorted sperm were

5   collected in a 50-ml plastic tube containing 2 ml of A-fraction freezing buffer for each extender; 15×10^6 total sorted sperm (25 ml) for each treatment were collected and incubated for 1 h at 22°C to simulate longer sorting.

b)   *Preparation for Freezing*. Diluted sperm were then cooled to 5°C over 90

10   minutes. An equal volume of appropriate B-fraction extender was added stepwise (2x) at 15-minute intervals to each 50-ml plastic tube containing sorted sperm. Aliquots of 25 ml/extender treatment were concentrated by centrifugation for 20 minutes at 850 x g in a refrigerated centrifuge. The supernatant was removed leaving a 600 µl sperm pellet, which was suspended by gentle vortexing for 15 seconds. No additional extender was

15   added to the sperm pellet since the suspension containing the pellet already contained glycerol. The concentration of the sperm suspension was approximately 20×10^6/ml. A non-stained, non-sorted control for each bull was prepared at 20×10^6 sperm/ml in egg-yolk-Tris extender containing 6% glycerol. The control was placed in a 5°C cold room while bulk-sorting occurred.

20
c)   *Equilibration and Freezing*. All control and bulk-sorted sperm were packaged and frozen at the same time. Sperm were packaged into 0.25-ml polyvinylchloride straws, equilibrated for about 3 h to about 6 h at 5°C and then frozen in static liquid nitrogen vapor.

25   3.   *Evaluation of Post-Thaw Motility*. Thawing and post-thaw evaluations of sperm were done as described for Example 2 with the exception that progressive motility was evaluated 0.5 and 2.0 h after incubation.

4.   *Statistical Analysis*. A general description of statistical analyses is provided in

30   Example 2. Specifically, treatment effects were evaluated via separate analyses of variance for each incubation time; the model included bull and extender in the main plot and observer and

36

related interactions in the subplot. Differences in means were determined by the least significant difference test.

5.   *Results*. Extender affected (P<0.05) progressive motility of sperm after 0.5 or 2 h
post-thaw incubation (Table 7). At 0.5 h, WET plus SDS resulted in lower motility than Tris with SDS. At 2 h, all treatments with bulk-sorted sperm were worse than the non-sorted control sperm. There were significant bull and observer effects (P<0.01) at both incubation times, but no observer by treatment interactions.

Table 7. Effect of extender on post-thaw progressive motility (%)

Extender	Incubation at 37°C	
	0.5 h	2 h
Tris (non-sort)	42^a	41^a
Tris w/o SDS	$40^{a,b}$	35^b
Tris w/SDS	42^a	37^b
WET w/o SDS	$40^{a,b}$	35^b
WET w/SDS	38^b	35^b
S.E.[c]	1.0	1.2

[a,b] Means within columns without common superscripts differ (P<0.05).

[c] $\sqrt{error\ mean\ square\ of\ ANOVA} \div \sqrt{N}$

6.   *Conclusion*. The inclusion of SDS in Tris or WET extenders did not benefit sperm quality as determined by visual estimates of motility after thawing. Also, results using WET and Tris extenders were similar; hence, WET appeared as efficacious as Tris for cryopreserving sorted bovine sperm.

37

Example 6

Quality of Sperm Sexed by Flow Sorting for Field Trials

Objective: to evaluate post-thaw quality of sorted sperm based on acrosomal integrity.

5   1.   *Collection of Source Sample*. Sperm of 3 bulls were collected and prepared as described in Example 1A.

    2.   *Methods*. Sorted and non-sorted control sperm from the same ejaculate were stained, processed, and sorted as described in Example 2 except the sperm were sorted for sex-

10 type at a 90% purity level. Sorted sperm were collected to a volume of approximately 20 ml and were cooled to 5°C for 90 minutes (0.2°C/min). After cooling, an equal volume of egg yolk-Tris B extender (see Example 2) was added to the sorted sperm in 2 equal volumes at 15-minute intervals. Centrifugation and aspiration of the supernatant were achieved as described in Example 5. After centrifugation and aspiration, egg yolk-Tris extender containing 6% glycerol

15 (v/v) was added to the sperm pellet to bring the concentration of sperm to about 20×10^6 /ml. Freezing and thawing were done as described in Example 2 except that equilibration time was about 3 h.

    3.   *Evaluation of Post-Thaw Motility*. Visual estimates of the percentage of

20 progressively motile sperm at 37°C were made approximately 10 minutes after thawing. The acrosomal integrity of sperm was assessed using differential interference-contrast microscopy (x1000) after 2 h of incubation at 37°C. Sperm were treated with 40 mM sodium fluoride, a wet was smear made, and 100 sperm per treatment were examined. Acrosomes were classified as: (a) intact acrosome, (b) swollen or damaged acrosome, or (c) missing acrosome (non-intact).

25
    4.   *Statistical Analysis*. The data analyzed were from 19 different freeze dates balanced across 3 bulls used in field trials. Treatment effects (sort vs. control) were evaluated via analysis of variance with bulls as a fixed effect.

    5.   *Results*. The percentage of progressively motile sperm post-thaw was

30 significantly higher ($P<0.05$) for non-sorted sperm (50%) than for sorted sperm (46%; Table 8), despite removal of dead sperm during sorting. However, the percentage of sperm with an intact

38

acrosome was not different. Sorting increased the percentage of sperm missing an acrosome, but also reduced the percentage of sperm with a damaged acrosome, relative to control sperm (P<0.05). There were significant differences among bulls for percent of intact acrosomes (P<0.05), percent of non-intact acrosomes (P<0.01), and post-thaw progressive motility

5 (P<0.01). There was a bull by sorting effect for post-thaw motility (p<0.01) but not for the other responses. From bulls A and B, differences in post-thaw motility between sorted and unsorted sperm were near zero; for bull C, sorted sperm were 10 percentage points (19%) lower in motility than control sperm.

10

Table 8.   Effect of sorting on post-thaw motility (%) and acrosomal status (%).

| | Acrosomal status | | | |
	Intact	Damaged	Non-intact	Post-thaw motility
Control	64[a]	20[a]	15[a]	50[a]
Sorted	65[a]	14[b]	21[b]	46[b]

[a,b] Column means with different superscripts differ (P<0.05).

6. *Conclusion*. Visual estimates of progressive motility for sorted, frozen sperm on average were slightly lower (4 percentage points; 8%) than for control sperm, although this

20 difference was larger for one bull. These evaluations were made approximately 10 minutes after thawing. The small average difference is consistent with that for non-intact acrosomes after 2 h of incubation. Sperm with a damaged or missing acrosome are likely to be immotile. The increased percentage of sperm with a non-intact acrosome, for sorted samples, indicates damage associated with sorting or with cryopreservation before or after actual sorting. Presumably,

25 sorting converted damaged acrosomes to missing acrosomes. Based on standard procedures for evaluation of sperm quality, there is no basis for assuming that fertilizing potential of these flow-sorted sperm should be severely compromised for most bulls.

30

Example 7

Sex-Selection and Cryopreservation of Bull Sperm Using 20% Egg Yolk-Tris Extender
Objective: to provide a protocol for the cryopreservation of flow-sorted bull sperm.

5   1.   *Collection and Ejaculate Assessment*. Collect and prepare ejaculates as described in Example 1A. Select ejaculates from those bulls with >75% morphologically normal sperm. Visually estimate the percentage of progressively motile sperm (ejaculates that have progressive motility >60% are best for sorting). Add antibiotics to raw semen as follows: tylosin at a final concentration 100 µg/ml, gentamicin at a final concentration of 500 µg/ml, and linco-spectin at a
10   final concentration of 300/600 µg/ml.

2.   *Staining and Preparation for Sort*. Following the addition of the antibiotics to the raw semen sample, allow 15-20 minutes before staining. Stain samples as described in Example 2.

15

3.   *Sorting*. Sort for both X- and Y- type sperm, setting the sorting gates for 90% purity. Sort sperm into 50-ml Falcon tubes containing 2 ml 20% egg yolk-Tris A-fraction extender (see Example 2) until each tube contains a maximum of 20 ml total volume (or a maximum of 2 h per sort) and final sorted sperm concentration is 6×10^5/ml. Note that
20   additional 20% egg yolk-Tris-A fraction catch buffer must be added after the sort and prior to cooling so that the final percentage of egg yolk is at least 3%.

4.   *Preparation for Freezing*. Following the sort, cool the sorted samples to 5°C over a period of 90 minutes. After cooling, add 20% egg yolk-Tris B-fraction extender (see Example
25   2) stepwise (2X) at 15 minutes intervals. The final volume of Tris B-fraction extender added to the sperm sample should be equal to the volume of Tris A-fraction extender. The total volume of sperm sample after the Tris B-fraction extender is added should not exceed 27 ml total volume.

After the Tris B-fraction extender is added to the sperm sample, concentrate the
30   sample by centrifugation for 20 minutes at 850 × g. Aspirate the supernatant leaving approximately 150 µl sperm pellet. Resuspend the sperm and pool the sperm for each individual bull.

40

5.   *Freezing*. Add complete egg yolk-Tris extender (6% glycerol) to achieve a final sperm concentration of 20×10^6/ml. Package the extended sperm into 0.25-ml polyvinylchloride straws for freezing as described in Example 2.

5

Example 8

Evaluation of the Fertility of Flow-sorted, Frozen Bull Sperm in Field Studies

MATERIALS AND METHODS

10   *Semen Collection and Processing*

Semen from young bulls of unknown fertility was collected via artificial vagina (see Example 1A). After determining sperm concentration with a spectrophotometer and subjective evaluation of progressive sperm motility, semen was processed and sorted as described in Example 2 except that the sperm were sorted by sex-type at 90% purity using a laser incident 15 power of about 135 to about 150 mW. Processing and freezing was achieved as in Example 2 except that the equilibration time was about 3 h. Cornell Universal Extender (Seidel GE Jr., Theriogenology 1997; 48:1255-1264) was used for liquid semen in field trials 1, 2, and 3. For frozen semen in field trials 2 and 3, the extender used was 2.9% Na citrate + 20% egg yolk with a final glycerol concentration of 7% (see Example 1). For field trials 4 through 11, sperm were 20 frozen in a Tris-based extender composed of 200 mM Tris, 65 mM citric acid, 56 mM fructose, 20% egg yolk, and a final glycerol concentration of 6% (see Example 2). The sheath fluid used in the flow cytometer was 2.9% Na citrate (see Example 4) for trials 1, 2, and 3, and a Tris buffer for the remaining trials (see Example 2).

Sperm were packaged in 0.25-mi French straws in columns as small as 50 μl in the center 25 of the straw. To minimize dilution effects, low volumes were used so there were at least 10^7 sperm/ml. In most trials, a column of extender without sperm was aspirated into the straw first to wet the cotton plug, followed by a small column of air, and then the sexed sperm. When sperm were frozen, one straw from each batch was thawed in 35°C water for 30 sec for quality control, and batches with less than 25% progressive motility post thaw were discarded. A sample of 30 sexed sperm from each batch was sonicated and analyzed by flow cytometry to determine the accuracy of sexing.

41

Heifer Management and Artificial Insemination

The heifers used were in 6 widely scattered production units with different management practices. Seasonal and breed differences contributed further to the heterogeneity of the experiments (Table 9). Insofar as possible, treatments and controls were alternated systematically within bulls within inseminators as heifers entered the insemination facilities.

Estrus was synchronized in one of 4 ways (Table 9): (1) 500 mg of melengesterol acetate (MGA) fed daily in 2.3 kg of grain for 14 days followed by an i.m. injection of 25 mg prostaglandin $F_2\alpha$ (Lutalyse, Upjohn, Kalamazoo, MI, USA) 17, 18 or 19 days after the last day of feeding MGA (MGA/PG); (2) a single injection of 25 mg of prostaglandin $F_2\alpha$ (PG); (3) 20 or 25 mg of prostaglandin $F_2\alpha$ injected i.m. at 12-day intervals (PG/PG) or (4) 50 or 100 μg of GnRH injected i.m., followed by 25 mg of prostaglandin $F_2\alpha$ 7 days later (GnRH/PG).

Heifers were inspected visually for standing estrus mornings and evenings, but inseminated only in the evenings after 16:00, approximately ½ or 1 day after onset of estrus. Insemination was either into the uterine body conventionally, or half into each uterine horn using atraumatic embryo transfer sheaths (IMV, Minneapolis, MN, USA). In the latter case, semen was deposited past the greater curvature of the uterine horn as far anterior as could be accomplished without trauma, identically to nonsurgical embryo transfer. In most cases, semen was deposited between the anterior third and mid-cornua.

Most experiments included a frozen sperm control inseminated into the uterine body with 20 or 40 x 10^6 sperm/dose from the same bulls used for sperm sorted for sex-type ("sexed"). This control served as a composite estimate of the intrinsic, normal fertility of the heifers under the specific field-trial conditions as well as the fertility of the bulls used and the skills of the inseminators. Some trials also included a low-dose, unsexed control group. Sometimes numbers of control inseminations were planned to be ½ or ⅔ the number used for each treatment to obtain more information on sexed sperm. Frozen sexed and control sperm were thawed for 20 to 30 sec in a 35 to 37°C water bath. Various other details are summarized in Table 9.

Pregnancy was diagnosed by ultrasound 28 to 37 d post insemination and/or 56 to 92 d post-insemination, at which time fetal sex was determined in most trials, as described in Curran, S., Theriogenology 1991; 36:809-814, without the operator's knowing insemination treatments or controls. Sexes of calves born were nearly identical to the fetal-sex diagnosis. Data were

42

analyzed by single-degree-of-freedom Chi square corrected for continuity; 2-tail tests were used unless 1-tail is specified. Fewer than 5% of the inseminations were culled due to errors of insemination treatment, frank infection of the reproductive tract, failure to traverse the cervix, etc. Decisions to cull animals from experiments were made shortly after insemination and were

5   never based on the pregnancy diagnosis.

10

15

20

25

30

43

Table 9. Procedural details of field trials

Trial	Insemination dates	Breeds of heifers	Bulls used	Inseminators	Estrus synchronization	Comments
1	5/20-23, 1997	Angus	N1, N2, AN4	A, B	MGA/PG	Included low-dose controls
2	2/18-5/22, 1998	Angus crossbred	N3, N4, N5, N6	C, D	PG/PG	Low dose but no normal-dose controls; some heifers pregnant and aborted when synchronized
3	6/2-6/5, 1998	Angus	AN4, AN5, N7, N8	B, D	MGA/PG	
4	2/10-13, 1999	Holstein	J2, J4	C, D	PG	Very severe mud, snow, wind, and cold, driving rain
5	2/24-26, 1999	Holstein	J2, J4, J5	C, B, D	PG/PG	
6	4/14-16, 1999	Holstein	J2, J3, J4, J5	C, D	PG	Some heifers were reproductive culls
7	4/27-5/1, 1999	Hereford& Angus crossbred	AN1, AN4	C	MGA/PG	Semen for 1 bull shipped 6 h before sorting; severe weather
8	4/21-23, 1999	Angus crossbred	H1, H2	E	MGA/PG	Feedlot heifers
9	5/5-8, 1999	Red Angus	AR1, AR2	C, F	MGA/PG	
10	5/31-6/2, 1999	Angus	AN4, AN7, AN8	B, D	GnRH/ PG	
11	7/28-30, 1999	Holstein	H2, H3	C, D	PG/PG	First replicate available in a much larger trial

44

RESULTS AND DISCUSSION

The data presented are from 11 consecutive, heterogeneous field trials, constrained by logistical aspects of the studies, such as having to match bulls to genetic needs of the herds, unavailability of fertility information on bulls, limited numbers of heifers, unavailability of the

5 same inseminators across trials, severe weather in some trials, limited amounts of sexed semen in early trials, 2 sets of heifers in which some turned out to be pregnant up to about 55 days at the time of estrus synchronization, etc. Up to 4 bulls and 3 inseminators were involved with each trial; this enabled us to sample populations to ensure that results applied to more than one bull or technician; however, insufficient data were produced to evaluate bull-to-bull differences in

10 fertility rigorously.

Most sets of heifers were from breeding herds located 140 to 250 km from our laboratory. There were no significant differences in pregnancy rates between inseminators in any trial, but numbers of breedings per inseminator were low, and differences likely would be detected with larger numbers of inseminations.

15 Estrus synchronization methods were not compared within trials, so it was not possible to compare pregnancy rates among these methods. Pregnancy rates appeared to be satisfactory for all four synchronization procedures used.

Since inseminations were done once a day, heifers in estrus evenings were inseminated approximately 24 h after estrus was detected. The pregnancy rate for these heifers with sexed

20 sperm pooled over all trials was 203/414 (49.0%), which was not significantly different (P>0.1) from that of heifers in estrus mornings and thus inseminated half a day after estrus detection 266/586 (45.4%). This tendency for higher fertility with later insemination is in agreement with findings from other research that it is preferable to inseminate later than normally recommended with lower fertility bulls, when low sperm numbers are used, or when conditions are otherwise

25 suboptimal.

Pregnancy rates by treatments and, when available, fetal or calf sex are presented in Tables 10 to 20. The objective was to obtain female offspring, except in trial 8; accuracy was 95%, 83%, 90%, 83%, 82%, and 94% in Trials 1, 3, 8, 9, 10, and 11, respectively. In the remainder of the trials, fetal or birth sexes were not available because of timing of pregnancy

30 diagnosis, unavailability of persons skilled in sexing fetuses, and/or because calves have not yet

45

been born. This was not a major concern because the main objective of this research was to determine fertility of flow-sorted sperm inseminated at low doses.

The accuracy of sexing can be adjusted to virtually any level desired between 50 and 95+% by adjusting the sorting parameters. However, higher accuracy results in lower numbers of sperm sorted per unit time, particularly for Y-chromosome sperm. 90% accuracy is sufficient for routine work.

The main findings from each field trial will be summarized in turn. Note that total sperm numbers are given in table headings; numbers of progressively motile sperm usually were 30 to 50% of these values. Field trial 1 (Table 10) confirmed that pregnancy rates with uterine horn insemination using low numbers of unsexed sperm were similar to controls with normal sperm numbers. The day 64 to 67 pregnancy rate with unfrozen sexed sperm (42%) was 12 percentage points below the unsexed liquid control with sperm diluted, stained, and centrifuged identically to the sorted sperm. Accuracy of sexing was 95%; the sex of calves born from sexed sperm matched the sex diagnosis of fetuses exactly; there was one mistake in sexing fetuses of controls. There were no abortions between 2 months of gestation and term, and all 19 calves from the sexed sperm treatment were normal and survived. For the sexed semen treatment, the 2-month pregnancy rates for bulls N1, N2, and N3 were 41, 44, and 40%, respectively; 39% (13/33) of heifers in estrus in the morning and 50% (6/12) in estrus in the evening became pregnant.

Table 10. Results of field trial 1 -- Angus heifers in Wyoming, 1997

Treatment/site	No. sperm	No. heifers	No. pregnant day 31 to 33	No. pregnant day 64 to 67	No. ♀ calves
Sexed, 5°C/horns	3×10^5	45	20 (44%)	19 (42%)	18 (95%)[a]
Control, 5°C/horns	3×10^5	28	15 (54%)	15 (54%)	5 (53%)[b]
Frozen, control/body	40×10^6	29	16 (55%)	15 (52%)	11 (73%)[a,b]

[a,b] Sex ratios without common superscripts differ (P<0.02).

Field trial 2 (Table 11) provided the first evidence that results with sexed, frozen sperm are similar to sexed, unfrozen sperm if adjustment is made for numbers of sperm killed during cryopreservation. There also was no difference in pregnancy rates between sexed sperm stored at 5 versus 18°C. Pregnancy rates at 2+ months after insemination for sexed semen from individual
5   bulls ranged from 22 to 42% pregnant (P>0.05). Embryonic loss between 1 and 2 months of gestation was very similar for sexed and control pregnancies. Calving data were available from 39 heifers from this trial; each of these heifers (30 sexed pregnancies, 9 controls) pregnant at 2 months calved after a normal-length gestation.

10   Table 11. Results of field trial 2--Crossbred beef heifers in Colorado, 1998

Treatment/site	No. sperm	No. heifers	No. pregnant day 30 to 35[a]	No. pregnant day 59 to 92[a]
Control, 5°C/horns	5×10^5	58	27 (47%)	24 (41%)
Sexed, 5°C/horns	5×10^5	51	17 (33%)	16 (31%)
Sexed, 18°C/horns	5×10^5	46	16 (35%)	12 (26%)
Sexed, frozen/horns	1×10^6	87	29 (33%)	28 (32%)

[a] No significant differences, χ^2.

20   Field trial 3 (Table 12) confirmed that sexed, frozen sperm results in reasonable pregnancy rates. The accuracy of sexing sperm was confirmed again; however, there were 4 errors in sexing fetuses relative to the calves born; the actual sexes of calves born are presented. Again, there were no abortions between 2 months of gestation and term. Pregnancy rates averaged over sexed, unfrozen and sexed, frozen sperm for bulls N8, N9, AN5, and AN4 were
25   24, 31, 50, and 60%, respectively (P<0.1).

30

Table 12. Results of field trial 3 -- Angus heifers in Wyoming, 1998

Treatment/site	No. sperm	No. heifers	No. pregnant day 62 to 65	No. ♀ calves
Sexed, 18°C/horns	5×10^5	37	11 (30%)[a]	10 (91%)[c]
Sexed, frozen/horns	1×10^6	35	18 (51%)[a,b]	14 (78%)[c]
Frozen, control/body	40×10^6	37	27 (73%)[b]	16 (59%)[d]

[a,b] Means without common superscripts differ $P<0.05$.
[c,d] The percentage of ♀ calves from the sexed treatments (83%) differed from the control group, $P<0.05$, 1-tail, χ^2.

Field trials 4, 5, and 6 (Tables 13, 14, 15) were done at the same location with 3 different groups of heifers. Unfortunately, it was not possible to replicate each trial similarly due to vagaries of field trials, such as scheduling personnel, availability of sexed semen from each bull, etc. The widely different pregnancy rates between trials 5 and 6 illustrate that conditions were different among trials. Some of the heifers in trial 6 were available because they failed to get pregnant after a month of natural mating. Under conditions of these trials, pregnancy rates were very similar between 1.5 and 3.0 x 10^6 sexed, frozen sperm/dose. Furthermore, there was no advantage to uterine-horn insemination. There were no significant differences ($P>0.05$) in pregnancy rates among bulls except in Trial 5 in which the pregnancy rate of J2, 20/28 (71%), was higher than that of J4, 15/39 (38%) ($P<0.05$). This difference was not consistent from trial to trial, as J4 had numerically but not significantly ($P>0.1$) higher pregnancy rates than J2 in Trials 4 and 6.

Table 13. Results of field trial 4 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 30 to 33	No. pregnant day 64 to 67*
Sexed, frozen/body	1.5×10^6	55	36 (65%)[a,b]	36 (65%)[a,b]
Sexed, frozen/body	3×10^6	52	27 (52%)[a]	26 (50%)[a]
Control, frozen/body	20×10^6	55	45 (82%)[b]	43 (78%)[b]

[a,b] Means without common superscripts differ (P<0.01).
* Six heifers pregnant at d 30 to 33 were sold before the second pregnancy diagnosis; these were assumed to have remained pregnant.

Table 14. Results of field trial 5 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 33 to 35[a]	No. pregnant day 60 to 62[a]
Sexed, frozen/body	1.5×10^6	23	12 (52%)	12 (52%)
Sexed, frozen/body	3.0×10^6	25	15 (60%)	14 (56%)
Sexed, frozen/horns	1.5×10^6	25	15 (60%)	12 (48%)
Sexed, frozen/horns	3.0×10^6	25	17 (68%)	15 (60%)
Control, frozen/body	20×10^6	30	20 (67%)	19 (63%)

[a] No significant differences.

Table 15. Results of field trial 6 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 31 to 34	No. pregnant day 60 to 63
Sexed, frozen/body	1.5×10^6	27	11 (41%)[a]	9 (33%)[a]
Sexed, frozen/body	3.0×10^6	25	10 (40%)[a]	9 (36%)[a]
Sexed, frozen/horns	1.5×10^6	24	8 (33%)[a]	7 (29%)[a]
Sexed, frozen/horns	3.0×10^6	24	10 (42%)[a]	8 (33%)[a]
Control, frozen/body	20×10^6	24	18 (75%)[b]	17 (71%)[b]

[a,b] Means without common superscripts differ ($P<0.05$).

For trial 7 (Table 16), only one inseminator was available due to rescheduling. This is the only trial that showed a convincing advantage of uterine-horn over uterine-body insemination. For this inseminator under the conditions of the trial, 55% more heifers (22 percentage points) became pregnant with sexed, frozen semen inseminated into the uterine horns than into the uterine body. The true difference could be smaller because there are wide confidence intervals on these means. In all the other trials (5, 6, 9, and 11) in which body- and horn-insemination were compared, pregnancy rates were very similar for both methods for this technician as well as for other technicians.

Semen from one of the bulls used in Trial 7 was shipped without dilution from Montana by air in an insulated box at ~20°C before sorting; shipping time was 6 h. Pregnancy rates for the sexed sperm from the two bulls were virtually identical, 49% for the unshipped and 52% for the shipped semen. Semen was not diluted with extender and not cooled for shipping because staining properties of sperm with Hoechst 33342 are altered by dilution with extenders. Furthermore, in other studies (see Example 4), storing semen neat at ambient temperature between collection and flow-sorting was found to be superior to diluting it.

Table 16. Results of field trial 7 -- Crossbred beef heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 33 to 37
Sexed, frozen/body	1.5×10^6	86	34 (40%)[a]
Sexed, frozen/horns	1.5×10^6	86	53 (62%)[b]
Control, frozen/body	20×10^6	35	18 (51%)[a,b]

[a,b] Means without common superscripts differ (P<0.01).

Field trial 8 (Table 17) concerned feedlot heifers not implanted with growth promotants; at the time pregnancy was diagnosed they were aborted, so calving data was not available. This experiment illustrates that efficacious sexing also can be done in the male direction. Pregnancy rates for the 2 bulls were 50 and 61%.

Table 17. Results of field trial 8 -- Angus heifers in Nebraska, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant[a] day 74 to 76	No. ♂ fetuses
Sexed, frozen, 72 mW laser/body	1×10^6	18	7 (39%)	6 (86%)
Sexed, frozen, 135 mW laser/body	1×10^6	18	13 (78%)	12 (92%)

[a] No significant differences.

Field trial 9 (Table 18) was the only trial to show a convincing advantage of 3.0 versus 1.5×10^6 sexed, frozen sperm/insemination dose. This advantage was true for both inseminators. Pregnancy rates for sexed sperm from the 2 bulls were 62 and 75%.

5

Table 18. Results of field trial 9 -- Red Angus heifers in Nebraska, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 60 to 63[a]	No. ♀ fetuses
Sexed, frozen/body	1.5×10^6	15	8 (53%)	7 (88%)
Sexed, frozen/body	3.0×10^6	14	12 (86%)	9 (75%)
Sexed, frozen/horns	1.5×10^6	16	9 (56%)	7 (78%)
Sexed, frozen/horns	3.0×10^6	16	12 (75%)	11 (92%)
Control, frozen/body	20×10^6	30	21 (70%)	13 (62%)

[a] 3.0×10^6 sexed sperm had a higher pregnancy rate (80%) than 1.5×10^6 sexed sperm (55%), $P<0.05$, 1-tail χ^2.

10

15

Pregnancy rates in field trial 10 (Table 19) with sexed, frozen semen, were similar to controls; the accuracy of sexing sperm on this trial was only 82%, which, however, is not significantly different from the targeted 90% accuracy. Pregnancy rates for sexed semen were 54, 66, and 50% for bulls AN4, AN7, and AN8, respectively ($P>0.1$). Eighteen of the heifers inseminated in this trial were the calves resulting from sexed sperm in field trial 1.

20

Table 19. Results of field trial 10 -- Angus heifers in Wyoming, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 61 to 63[a]	No. ♀ fetuses
Sexed, frozen/body	1×10^6	44	26 (59%)	23 (85%)
Sexed, frozen/body	3×10^6	43	23 (53%)	17 (74%)
Control, frozen/body	20×10^6	35	20 (57%)	12 (57%)

[a] No significant differences.

25

30

Table 20. Results of field trial 11 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 28 to 30[a]	No. pregnant day 56 to 58[a,b]
Sexed, frozen/body	1×10^6	12	8 (67%)	7 (58%)
Sexed, frozen/body	3×10^6	12	6 (50%)	4 (33%)
Sexed, frozen/horns	1×10^6	7	4 (57%)	4 (57%)
Sexed, frozen/horns	3×10^6	7	4 (57%)	4 (57%)
Control, frozen/body	20×10^6	9	4 (44%)	3 (33%)

[a] No significant differences, χ^2.
[b] 16 of 17 (94%) fetuses from the sexed semen treatments were female; 2 were too deep in the body cavity to sex with ultrasound.

Data from trials were combined in which treatments were identical except 1 x 10 and 1.5 x 10^6 sperm doses were pooled (Table 21).

Table 21. Meta-summary from combining trials with sexed, frozen semen and frozen controls.

Trials combined	Sperm no./site	No. heifers	No. pregnant
5, 6, 9, 11	$1.0 - 1.5 \times 10^6$/body	77	36 (47%)
	3.0×10^6/body	76	38 (50%)
	$1.0 - 1.5 \times 10^6$/horns	72	32 (44%)
	3.0×10^6/horns	72	39 (54%)
	20×10^6/body, control	93	61 (66%)
4, 5, 6, 9, 10, 11	$1.0 - 1.5 \times 10^6$/body	176	98 (56%)
	3.0×10^6/body	171	88 (51%)
	20×10^6/body, control	183	124 (68%)
5, 6, 7, 9, 11	1.5×10^6/body	163	70 (43%)
	1.5×10^6/horn	158	85 (54%)
	20×10^6/body, control	128	79 (62%)

Pregnancy rates with sexed sperm were generally 70-90% of unsexed controls within experiments with 7 to 20 times more sperm. This difference was less in the more recent trials, possibly reflecting improved sexing and sperm-processing procedures.

In some trials, heifers were examined for pregnancy by ultrasound at both 1 and 2 months after insemination. Pregnancy losses in this interval were similar (P>0.1) for sexed (23/261; 8.8%) versus control (9/145; 6.2%) sperm treatments, which is one measure that genetic damage due to sexing is minimal. Calving information was obtained from only a few of the pregnant

5   heifers because most cattle from the earlier trials were sold, and those from later trials have not calved yet. The population of calves produced to date from sexed semen appears to be no different from the population of controls.

10
CONCLUSION

Sex ratios in cattle can be distorted to about 90% of either sex by sorting sperm on the basis of DNA content with a flow cytometer/cell sorter followed by cryopreservation and relatively routine artificial insemination. Calves resulting from sexed sperm appear to be normal. For most bulls in these studies, pregnancy rates with 1.0 to 1.5 x 10^6 sexed, frozen sperm were 70

15   to 90% of unsexed controls with 20 or 40 x 10^6 frozen sperm inseminated conventionally. These results apply to well-managed heifers bred by well-trained inseminators using properly processed semen. There may be a small advantage to inseminating sexed sperm bilaterally into the uterine horns compared to standard uterine body insemination.

20

****

The present invention has of necessity been discussed herein by reference to certain specific methods and materials. It is to be understood that the discussion of these specific

25   methods and materials in no way constitutes any limitation on the scope of the present invention, which extends to any and all alternative materials and methods suitable for accomplishing the ends of the present invention.

All patents and publications described are herein incorporated by reference in their entirety.

30